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ScienceDirect
Developmental Biology 301 (2007) 309–326
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Review

Molecular control of secondary palate development

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Received for publication 12 May 2006; revised 24 July 2006; accepted 28 July 2006
Available online 5 August 2006

Abstract

Compared with the embryonic development of other organs, development of the secondary palate is seemingly simple. However, each step of palatogenesis, from initiation until completion, is subject to a tight molecular control that is governed by epithelial–mesenchymal interactions. The importance of a rigorous molecular regulation of palatogenesis is reflected when loss of function of a single protein generates cleft palate, a frequent malformation with a complex etiology. Genetic studies in humans and targeted mutations in mice have identified numerous factors that play key roles during palatogenesis. This review highlights the current understanding of the molecular and cellular mechanisms involved in normal and abnormal palate development with special respect to recent advances derived from studies of mouse models.
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Keywords: Palate development; Cleft palate; Epithelial–mesenchymal interactions; Mouse mutations; Signaling pathways; Palate patterning

Introduction

A major leap forward has been achieved from the superstition-ridden times when congenital malformations such as cleft lip ("hare lip") and cleft palate were regarded as the work of supernatural malefic forces. Attempts to compensate for those defects go back in time. It has thus been suggested that the Greek orator Demosthenes (384–323 B.C.) used pebbles as obturators to compensate for his cleft lip/palate in order to improve his speech (Bien, 1967). The last few decades have witnessed major improvements in the treatment of cleft palate. Yet, in addition to the need for a multidisciplinary lengthy treatment which is a burden to the affected individual, there may be long-term sequelae, including speech defects, velopharyngeal insufficiency or incompetence, palatal fistulae, and midfacial growth distortion. These aspects beg for further therapeutic improvements and a better understanding of the etiopathogenesis of cleft palate.

Cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO) occur in 1/500 to 1/1000 births worldwide, with CL/P being more frequent than CPO (Marazita, 2002). These orofacial clefts have been sub-categorized into syndromic and nonsyndromic forms. The majority of CL/P and CPO are nonsyndromic with an estimated genetic contribution of 20–50% (Marazita, 2002). More than 300 syndromic disorders have been described in which CL/P or CPO is a feature. These can occur as part of a Mendelian inheritance of alleles at a single genetic locus, whereas others are due to recurrent chromosomal rearrangements and teratogens (Marazita, 2002; Muenke, 2002). The emerging consensus for the etiology of CL/P and CPO is that of complexity, caused by both genetic and/or environmental factors. (Schutte and Murray, 1999; Marazita, 2002; Jugessur and Murray, 2005). Several genes implicated in Mendelian syndromic forms of CL/P seem also to play a role in the etiology of isolated (nonsyndromic) clefts. These include the homeobox gene *MSX1* (CL/P with hypodontia), the T-box gene *TBX22* (X-linked CP and ankyloglossia), and genes encoding the interferon regulatory factor 6 (*IRF6*), nectin-1 (*PVRL1*; polio virus receptor related 1) and the fibroblast growth factor receptor 1 (*FGFR1*) (Stanier and Moore, 2004; Jugessur and Murray, 2005; Rice, 2005). For comprehensive treatises of the pathogenesis, genetics, environmental risk factors and clinical care of orofacial clefting, the reader is referred to excellent recent reviews (Reisberg, 2000; Wilkie and Morriss-Kay, 2001; Marazita, 2002; Cobourne, 2004;

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0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.07.042

Murray and Schutte, 2004; Stanier and Moore, 2004; Jugessur and Murray, 2005; Rice, 2005).

While the different steps of embryonic development of the mammalian secondary palate (see below) were already established at the time the subject was reviewed by Peter (1924), the detailed biological events regulating palate development as well as the etiopathogenesis of CP are still not well understood despite decades of intensive research. The last two decades have witnessed an impressive sophistication in research methodologies and a profusion of genetically modified mouse models of diseases. Naturally, these have been implemented in studies of palate development and led to new discoveries and to the confirmation and/or refinement of earlier ones. This review thus aims to bring into focus current insights into the molecular and cellular mechanisms regulating secondary palate development and the key advances that have emanated from mouse studies.

Embryonic development

Development of the face and jaws is the product of growth and fusion of prominences (processes) and involves cell migration, proliferation, differentiation and apoptosis. These primordia consist of a mesenchymal core derived mainly from the cranial neural crest and of an ectodermally derived epithelial outer covering. Around embryonic day 10.5 in the mouse embryo (E10.5; corresponding to early 6th week of gestation in humans), the medial nasal processes which derive from the frontonasal process merge with each other and with the bilateral maxillary processes to form the upper lip and the primary palate. Merging of the bilateral mandibular processes across the midline produces the lower lip and the lower jaw. Around E11 in the mouse (6th week of gestation in humans), the earliest sign of secondary palate initiation is manifested as bilateral outgrowths, primordia of the palatal shelves (PS), which emerge from the inner part of the maxillary processes and extend antero-posteriorly along the lateral walls of the oropharynx (Fig. 1A). From E12.5–E14, the PS grow first vertically in the oral cavity (Figs. 1B–I), then elevate into a horizontal position (Figs. 1J–L) (E14.5–E15; gestation weeks 7–8 in humans) above the tongue. Further polarized growth ensures approximation of the opposing PS and their adherence along the medial edge epithelia (MEE), creating a transient multilayered epithelium, the midline epithelial seam (MES) (Figs. 2A–C). The progressive disappearance of the MES (Figs. 2D–F) allows the fusion of the PS along the midline (Figs. 2G, H). The PS also fuse with the primary palate anteriorly and with the nasal septum dorsally. Upon completion of palatogenesis, the early oronasal cavity becomes subdivided into an oral and a nasal cavity, a prerequisite for simultaneous breathing and feeding. Further differentiation of mesenchymal cells produces the palatal processes of the maxillary and palatine bones of the hard palate (Figs. 2I, J). The posterior-most extension of the secondary palate, the soft palate, is a complex muscular organ.

Compared with other organs such as the brain, lung and heart, palate development may seem simple. However, the different steps of palatogenesis are tightly regulated, and failure of PS growth, elevation, contact and fusion or failure of mesenchymal differentiation generate a CP. In addition, secondary palate development occurs in concert with the development of other oral and craniofacial components, implying that their impaired development can cause CP.

Mouse genetic mutations and cleft palate

Cleft of the secondary palate (CP) (Figs. 3A–D) has been reported in a growing number of mice carrying mutations in genes encoding transcription factors, growth and signaling molecules and their receptors, extracellular matrix components as well as intracellular effectors (Table 1). Several of these mutations generate CP following intrinsic disruptions in the cellular and molecular events controlling PS growth, elevation or fusion, whereas others cause CP as a secondary event following craniofacial bone and/or tongue anomalies.

Molecular control of palatal shelf growth

Targeted gene mutations in mice have revealed a number of molecular determinants of PS growth (Table 1). In these, the PS are hypoplastic and either remain in a vertical position, leading to a wide cleft, or manage to elevate but remain apart.

Organogenesis is governed by interactions between adjacent tissues layers. Organs as diverse as the lung, neural tube, tooth, hair and palate share several signaling pathways, although the developmental outcome is different. This emphasizes the notion of 'common notes—different melodies', where similar molecular networks are used during ontogeny of several organs but regulate different processes. Thus, insights gained from the biological events operating during embryogenesis of one organ can be used to shed light into those acting in other organs.

Early experimental studies indicated a role for epithelial–mesenchymal interactions in the regional specification of PS epithelia and growth of the PS (Tyler and Koch, 1977; Tyler and Pratt, 1980; Ferguson and Honig, 1984). More recent studies identified several molecular networks operating between the PS epithelium and mesenchyme during the different steps of palatogenesis. These include signaling molecules and growth factors such as Sonic hedgehog (Shh), members of the transforming growth factor β (Tgfβ) superfamily, including bone morphogenetic proteins (Bmps) and Tgfβs, fibroblast growth factors (Fgfs), their receptors, effectors and targets.

Transcription factors play fundamental roles in tissue patterning, growth and differentiation. *Msx1*, the LIM-homeobox containing *Lhx8*, the short stature homeobox *Shox2* and the *odd-skipped related2* (*Osr2*) genes have been shown to be expressed in the growing PS. Targeted mutations of these genes generate CP with minor or no craniofacial anomalies, indicating an intrinsic requirement of these factors during palatogenesis (Satokata and Maas, 1994; Zhao et al., 1999; Zhang et al., 2002; Lan et al., 2004; Yu et al., 2005). The CP in mice lacking *Msx1* ($Msx1^{-/-}$) has been shown to be caused by altered mesenchymal proliferation (Zhang et al., 2002). *Msx1* and *Msx2* genes are

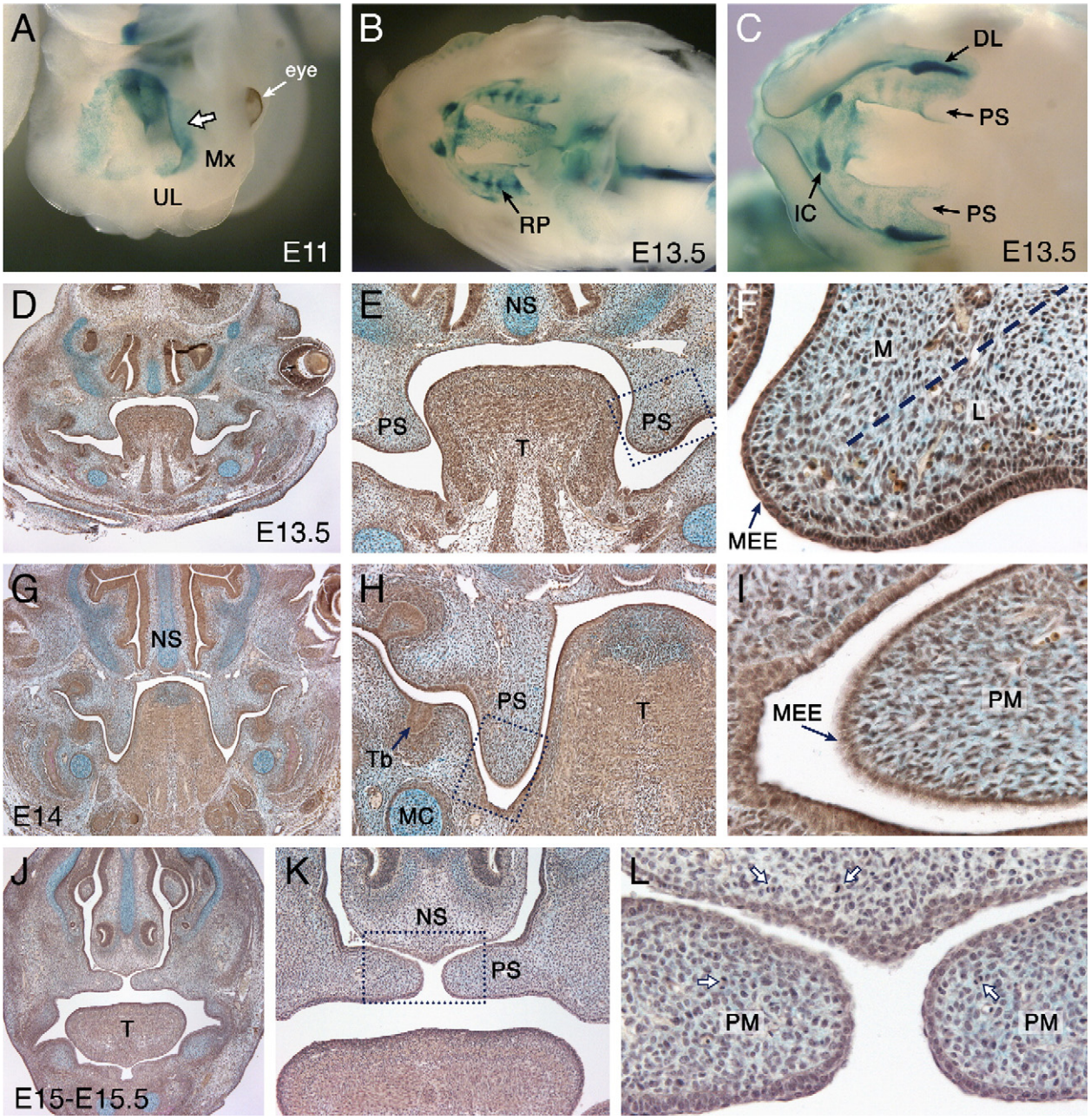

Fig. 1. Whole-mount β-galactosidase staining of developing palates from *ShhGFPCre/+*; *R26R/+* (A, B) and *K14-Cre/+*; *R26R/+* (C) embryos at E11 (A), E13.5 (B, C). The developing mandible and tongue were removed (A–C). In panel A, the head was tilted slightly to show the nascent palatal shelf (arrow). β-Galactosidase staining (dark blue) visualizes cells that express or have expressed *Shh* (A, B) or *Keratin 14* (C) as well as their progeny. Histological sections stained with the Alcian Blue-van Gieson method showing the developing palates of E13.5 (D–F), E14 (G–I) and E15–E15.5 (J–L) mouse embryos. Panels E, H, and K are high magnification views of panels D, G and J, respectively. Panels F, I and L are high magnification views of the indicated areas in panels E, H and K, respectively. The growing palatal shelves (PS) are vertical at E13.5 and E14. Between E15 and E15.5, the PS have assumed a horizontal position above the tongue. Arrows in panel L indicate mitotic figures in nasal septum (NS) and PS mesenchymal cells. DL, dental lamina; IC, developing incisor tooth; L, lateral side of the PS; M, medial side of the PS; MC, Meckel's cartilage; MEE, medial edge epithelium; Mx, developing maxilla; PM, palatal mesenchyme; RP, rugae palatinae; T, tongue; Tb, molar tooth bud; UL, upper lip. The blue color in the extracellular matrices of the PS and cartilage is due to Alcian blue staining of negatively charged macromolecules such as glycosaminoglycans (D–L).

bona fide targets of Bmp signaling in different developing embryonic sites including the tooth, cranial sutures, hair follicle and neural tube, where they act to regulate morphogenesis and differentiation (Vainio et al., 1993; Kim et al., 1998; Kulessa et al., 2000; Ramos and Robert, 2005). Further, in both the embryonic tooth and palate, *Msx1* has been shown to be necessary for expression of *Bmp4* and/or *Bmp2* (Zhang et al., 2000; 2002). Interestingly, exogenous Bmp4 or a mesenchymally expressed *Bmp4* transgene were capable to rescue the tooth developmental arrest and CP, respectively, in *Msx1*$^{-/-}$ mice (Bei et al., 2000; Zhang et al., 2002). Further elegant experiments (Zhang et al., 2002) indicated that Msx1 and Bmp4

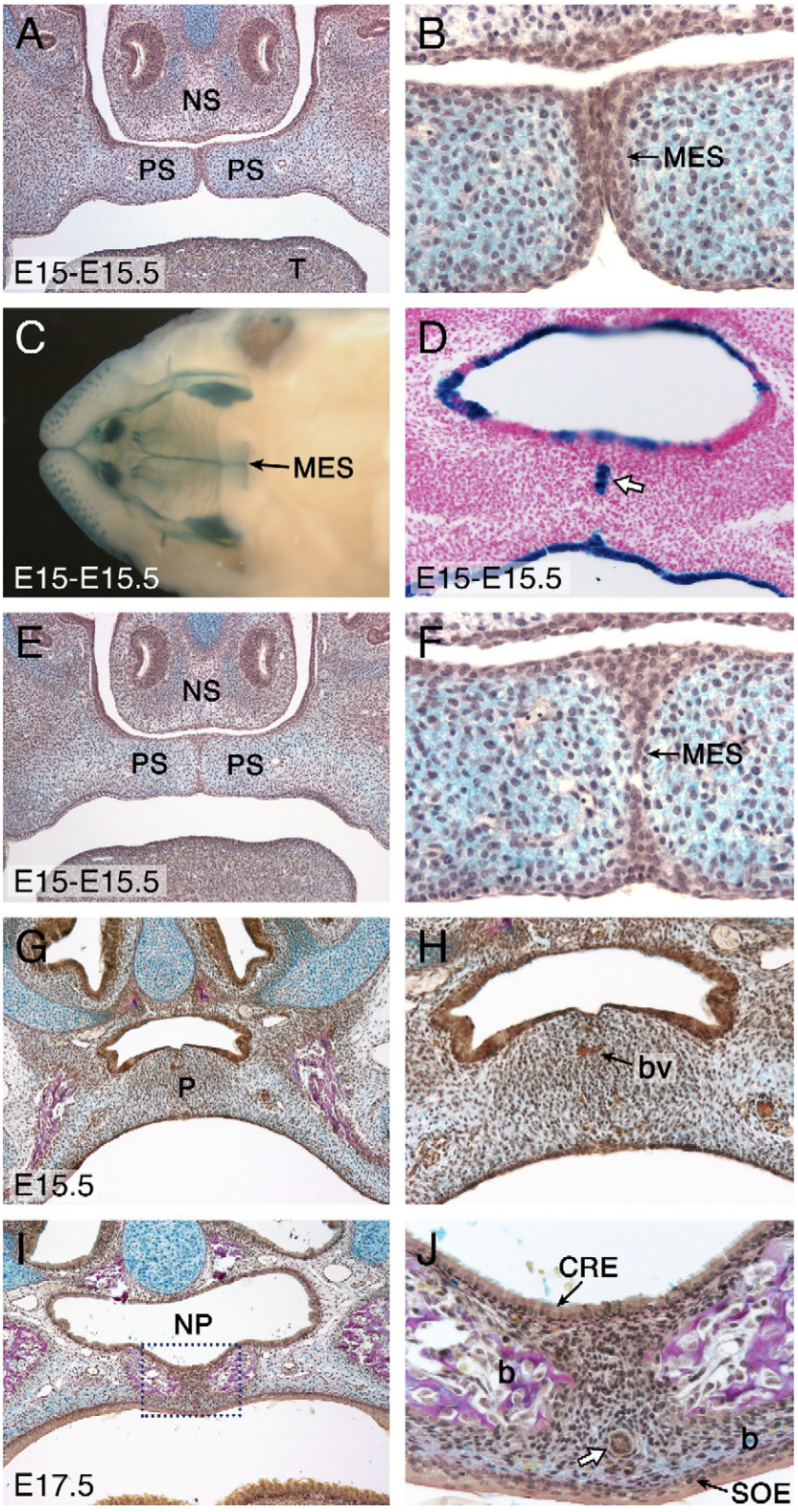

Fig. 2. Histological sections (A–B, E–J) stained with the Alcian Blue-van Gieson method showing the developing palate of mouse embryos at E15–E15.5 (A, B, E, F), E15.5 (G, H) and E17.5 (I, J). Sections in panels A, B, E and F are from the same specimen shown in Fig. 1J and have been taken at slightly more posterior levels to the section shown in Fig. 1J. Whole-mount (C) and tissue section (D) of developing palates from *K14-Cre*$^{/+}$; *R26R*$^{/+}$ embryos at E15–E15.5 showing β-galactosidase activity (dark blue color). In panel A, the opposing palatal shelves (PS) have just made contact with each other through their MEE, creating the medial epithelial seam (MES). Note the regressing MES (D–F). The fact that mesenchymal cells at the midline are β-galactosidase-negative rules out the occurrence of any epithelial–mesenchymal transformation of the MES. The white arrow indicates a β-galactosidase-positive epithelial island (D). Disappearance of the MES and establishment of mesenchymal confluence (G, H). Differentiation of the remaining epithelium of the palate into ciliated respiratory (CRE) and squamous oral (SOE) epithelia. The white arrow in panel H indicates an epithelial island, remnant of the MES, that will disappear later. b, developing palatal process of the palatine bone; bv, blood vessel; NP, nasopharynx; P, palate; T, tongue.

function in an autoregulatory loop in regulating mesenchymal proliferation in the anterior palate.

Recently, nestin-Cre-mediated removal of type I Bmp receptor (BmpR1A; Alk3) as well as Bmp4 activities demonstrated distinct functions for Bmp signaling in lip fusion and secondary palate development in mice (Liu et al., 2005). Ablation of BmpR1A function in both the epithelium and mesenchyme of lip and palate primordia was found to generate bilateral cleft lip and palate. Altered cell proliferation and misexpression of *Barx1* and *Pax9* in the palate as well as precocious cell death in the fusing lip seem to be the cause of the clefting in the *Bmpr1a* mutants. In these, expression of other important factors such as *Msx1*, *Tbx22* and *Osr2* was unchanged. However, conditional removal of Bmp4 activity resulted in isolated cleft lip (Liu et al., 2005). The latter phenotype seems at odds with the previously demonstrated important role for mesenchymal Bmp4 in the developing palate (Zhang et al., 2002). Further studies are necessary to provide an explanation for these differences. Keratin 14-Cre-mediated targeted mutation of *Bmpr1a*, which inactivates this receptor in ectodermally derived tissues, including tooth, skin and palatal epithelia, has been shown to affect tooth and hair follicle development. However, the palate seems to develop normally in mutant mice (Kobielak et al., 2003; Andl et al., 2004). Altogether, these observations indicate that BmpR1A functions primarily within the PS mesenchyme.

Targeted inactivation of *Osr2* indicates a role for this transcription factor in medio-lateral (see below) patterning of the PS. In $Osr2^{-/-}$ mice, the proliferation defects in the PS mesenchyme and the delayed elevation of the PS seem to be independent of Msx1, Bmp, Shh and Tbx22 inputs but may be linked to Pax9 and Osr1 function (Lan et al., 2004).

Other studies addressed the role of Fgf signaling during early palate development by analyzing mouse embryos lacking the functions of Fgf10 and FgfR2b (Rice et al., 2004; Alappat et al., 2005). In the $Fgf10^{-/-}$ and $Fgfr2b^{-/-}$ mutants, altered cell proliferation within both the PS mesenchyme and epithelium as well as increased apoptosis within the epithelium seem to be the primary causes of CP. Those studies also revealed an interesting epithelial–mesenchymal signaling loop. By signaling via its receptor FgfR2b in the PS epithelium, the mesenchymally derived Fgf10 brings not only about epithelial proliferation and survival but also induces expression of *Shh* within the epithelium. Shh, in turn, signals to the mesenchyme and stimulates cell proliferation (Rice et al., 2004).

In general, signaling activities are subject to tight spatio-temporal control, and in many instances too much or too little of a good thing can be detrimental to a developing organ. This is well illustrated in anomalies caused by deregulated Hedgehog (McMahon et al., 2003) and Fgf (Rice, 2005; Nie et al., 2006) signaling. While Fgf10/FgfR2b activity plays a crucial role during palatogenesis, it appears to be subject to a tight spatiotemporal regulation as recently shown in mice lacking *Shox2* (Yu et al., 2005). $Shox2^{-/-}$ mice (Yu et al., 2005) develop a very rare type of palatal clefting that may also be found in humans and other mammalians (Schüpbach, 1983); the soft palate is intact, whereas the hard palate is cleft. Abnormal proliferation and apoptosis are likely at the core of the clefting. Surprisingly, a number of protagonists implicated in palatogenesis, including *Msx1*, *Bmp4*, *Pax9*, *Lhx8*, *Osr2*, *Tgfβ3* and *Jag2*, were found to be expressed normally. In contrast, *Fgf10* and *Fgfr2c* were expressed at ectopic sites within the PS mesenchyme of the $Shox2^{-/-}$ mice (Yu et al., 2005). These studies re-emphasize the importance of a fine tuning of the timing and sites of signaling activities for normal development to take place.

Tgfβ peptides activate the membrane receptor serine/threonine kinase quaternary complex made of two type II and two type I receptors. The type I Tgfβ receptor Alk5 has been recently shown to play a key role in craniofacial and palate development (Dudas et al., 2006). The craniofacial anomalies of *Alk5* mutants were more severe than those in corresponding mutants lacking the function of the TGFβ type II receptor (TgfβRII) in cranial neural crest derivatives (Ito et al., 2003). Those striking differences have been suggested to be due to Alk5 function in mediating signalings by ligands other than Tgfβ1–3 and to the ability of Alk5 to function with type II receptors other than TgfβRII (Dudas et al., 2006). In contrast to embryos lacking *Tgfbr2* in the PS mesenchyme, which displays reduced cell proliferation (Ito et al., 2003), the *Alk5*-deficient PS mesenchyme seems to be hyperproliferative and to undergo massive apoptosis (Dudas et al., 2006), again pointing to differences in the signaling functions of these two receptors. In humans, abnormally high Tgfβ activity impinges upon palate formation as demonstrated in individuals bearing mutations in *TGFBR1* or *TGFBR2* (Loeys et al., 2005). These findings indicate that while signaling activities of type I and type II Tgfβ receptors are crucial, the amplitude of such signals must be tightly controlled for normal palatogenesis.

With the exception of the developing limb, organs consisting of an epithelium and a mesenchyme express the Hedgehog family members, *Shh* or *Indian hedgehog* (Ihh), in the epithelial compartment, whereas targets and effectors of the Hedgehog pathway are found in both tissue layers, indicating Shh and Ihh activities at a distance from their sources (McMahon et al., 2003). In the developing palate, Shh is produced in the PS epithelium, whereas its membrane receptor Patched1 (Ptc1) is present in both the epithelium and mesenchyme. The Hedgehog transcriptional effectors Gli1–3 are expressed in the PS mesenchyme (Rice et al., 2006) but are present at low levels in the PS epithelium as well (AGL, unpublished). Abrogation of Shh function in the palate epithelium generates CP. In contrast, epithelial loss of function of Smoothened (an obligatory and nonredundant component for all Hedgehog signaling) does not generate CP, implying that the PS mesenchyme is the major target for Shh action (Rice et al., 2004). However, this does not exclude the possibility of an indirect action of Shh on the PS epithelium via Shh-induced mesenchymal inputs. Shh has been shown to act as a powerful mitogen in numerous developmental and neoplastic contexts (McMahon et al., 2003). In vitro cultures showed that Shh stimulates PS mesenchymal proliferation (Rice et al., 2004). Other in vitro studies have shown that Shh induces/maintains Bmp2 expression, and that Bmp2 mediates Shh mitogenic effects on PS mesenchyme (Zhang et al., 2002).

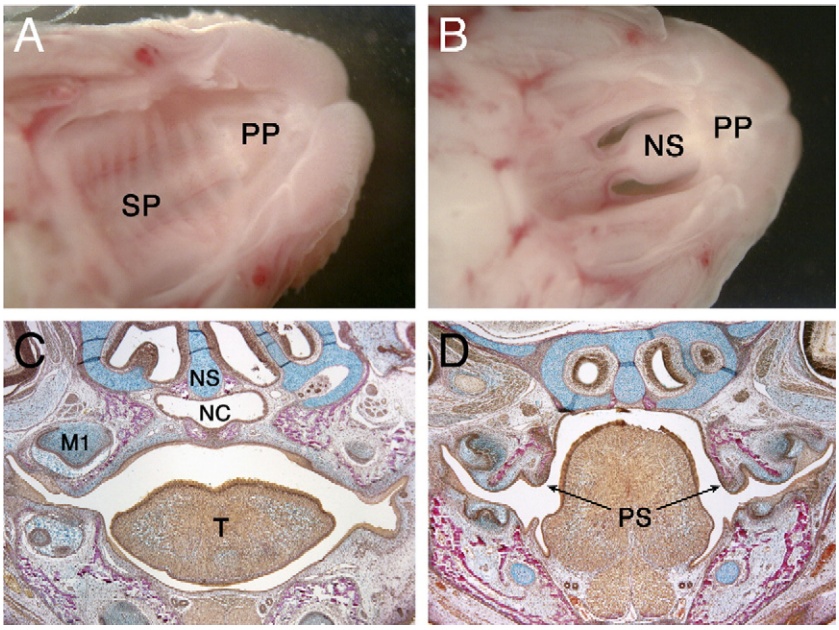

Fig. 3. View of the roof of the oral cavity showing the secondary (SP) and primary (PP) palates in a wild-type (A) and a *K14-Cre; Shh*$^{n/c}$ mutant (B) mouse fetuses at E17.5–E18. The *K14-Cre; Shh*$^{n/c}$ mutant, which lacks Shh function in the palate, develops a wide cleft of the secondary palate. Panels C and D are sections stained with Alcian Blue-van Gieson stain from the specimens shown in panels A and B, respectively. Note the severe hypoplasia of the palatal shelves (PS) which failed to elevate in the mutant (D). M1, upper first molar; NC, nasal cavity; NS, nasal septum; T, tongue.

Loss of the *Sall3* gene in mice generates palatal deficiency characterized by hypoplasia of the soft palate and epiglottis (Parrish et al., 2004). *Sall3*, which is a member of the *Spalt* gene family encoding putative transcription factors, is expressed in the palatal mesenchyme (Parrish et al., 2004). Interestingly, the *Spalt* genes have been shown to be downstream targets of Hedgehog signaling in both *Drosophila* and vertebrates (Koster et al., 1997; Sturtevant et al., 1997), and hypoplasia or absence of the epiglottis has been reported in humans with Pallister-Hall syndrome caused by *GLI3* mutations. Thus, interactions between the Hedgehog pathway and *Spalt* genes might occur during palatogenesis.

Mutations of the *p63* (*TP63*) gene encoding the transcription factor p63, a member of the p53 family, cause the three allelic disorders ectrodactyly ectodermal dysplasia-clefting syndrome 3, ankyloblepharon-ectodermal dysplasia-clefting syndrome and Rapp-Hodgkin syndrome (Celli et al., 1999; McGrath et al., 2001; Bougeard et al., 2003; Shotelersuk et al., 2005). Heterozygous *p63* mutations seem also to cause nonsyndromic CL/P (Leoyklang et al., 2006). The presence of at least six different isoforms of p63, some of which display opposing activities (Yang et al., 1998; van Bokhoven and Brunner, 2002), complicates analysis of p63 function and may underlie the wide phenotypic spectrum of anomalies in the above syndromes. p63 plays a pivotal role in epithelial development, where it regulates the expression of an array of factors that are essential for cell proliferation, integrity and survival (Mills et al., 1999; Yang et al., 1999; Koster et al., 2004; Carroll et al., 2006). Homozygous mice lacking p63 display limb, maxillary and palatal truncations and lack ectodermally derived appendages (Mills et al., 1999; Yang et al., 1999). The developing limb buds of p63 mutants lack a morphologically and molecularly distinct apical ectodermal ridge that is crucial for epithelial–mesenchymal interactions driving limb outgrowth (Mills et al., 1999; Yang et al., 1999). Altered epithelial–mesenchymal interactions may also underlie the CL/P in humans with p63 mutations. In the fusing ectoderm of the nasal processes of embryos lacking *Bmpr1a*, *p63* expression has been shown to be down-regulated (Liu et al., 2005), suggesting that *p63* is a target of Bmp signaling, echoing the findings in zebrafish (Bakkers et al., 2002). The exact mode of action of p63 during palatogenesis awaits further studies.

Recently, high-resolution breakpoint mapping techniques identified disruptions of the *SATB2* gene encoding a homeodomain protein in two de novo CPO-associated translocations on 2q32–q33 in humans. The breakpoints seem to result in functional haploinsufficiency (FitzPatrick et al., 2003). The mouse homolog of this gene is expressed in PS mesenchyme during its growth phase (FitzPatrick et al., 2003; Dobreva et al., 2006), and homozygous mice lacking the function of Satb2 indicate that this factor acts as a molecular node in regulating craniofacial patterning and osteoblast differentiation (Dobreva et al., 2006). In contrast to *SATB2* haploinsufficiency in humans, heterozygous mice with one functional *Satb2* allele are phenotypically normal, suggesting species-specific requirement for *Satb2* dosage. In the *Satb2*$^{-/-}$ embryos, the PS display peculiar bulges, indicating a patterning defect during the growth phase, and fail to elevate on time, probably as a consequence of hindrance by the tongue. While the reduced expression of *Lhx8* in the *Satb2* mutants (Dobreva et al., 2006) might be linked to palatal clefting it cannot account for the patterning defects, since in mutants lacking *Lhx8* which display a CPO (see below) the PS are devoid of patterning anomalies (Zhao et al., 1999).

Table 1
Genes implicated in cleft palate in mice

Genetic loss-of-function	Causes of cleft palate	References
Signaling proteins and receptors		
Activinb-A	Δ	Matzuk et al., 1995a,b
Activin receptor type II	Δ	Matzuk et al., 1995a
αv integrins	Palatal shelves elevate but fail to make contact	Bader et al., 1998
Bmpr1a (Alk3) nestin-Cre-mediated ablation)	Cell proliferation defects and altered anterior posterior patterning	Liu et al., 2005
Bmp type I receptor (Alk2) Wnt1-Cre-mediated ablation	Δ	Dudas et al., 2004b
Egfr	Failure of fusion of the palatal shelves (persistence of the MEE)	Miettinen et al., 1999
Et1	Δ	Kurihara et al., 1994
Ephb2; Ephb3	Hypoplastic palatal shelves	Orioli et al., 1996
Fgf10	Proliferation defects and increased apoptosis in palatal shelves; Loss of *Shh* expression, aberrant adhesion of palatal shelves with other oral epithelia	Rice et al., 2004; Alappat et al., 2005
Fgf18	Δ	Liu et al., 2002; Ohbayashi et al., 2002
Fgfr2b	Altered proliferation in palatal shelves	De Moerlooze et al., 2000; Rice et al., 2004
Follistatin	Δ	Matzuk et al., 1995c
Gabrb3	Palatal shelves elevate but fail to make contact	Homanics et al., 1997; Hagiwara et al., 2003
Jagged2	Aberrant adhesion between palatal shelf and oral epithelia secondary to altered differentiation of the epithelium of the tongue and mandible epithelium	Jiang et al., 1998; Casey et al., 2006
Pdgfc	Hypoplastic palatal shelves, delayed elevation and failure of fusion of palatal shelves	Ding et al., 2004
Pdgfc; Pdgfa compound mutants	Δ	Ding et al., 2004
Pdgfra (Wnt1-Cre-mediated ablation)	Δ	Tallquist and Soriano, 2003
Ryk	Δ	Halford et al., 2000
γRAR	Δ	Lohnes et al., 1993
Shh (K14-Cre-mediated ablation)	Altered proliferation and increased apoptosis in palatal shelves	Rice et al., 2004
Tgfb2	Δ	Sanford et al., 1997
Tgfb3	Failure of fusion of palatal shelves	Kaartinen et al., 1995; Proetzel et al., 1995
Tgfbr2 (Wnt1-Cre-mediated removal)	Proliferation defects of palatal mesenchyme	Ito et al., 2003
Tgfbr2 (K14-Cre-mediated removal)	Impaired palatal fusion (partial) due to lack of apoptosis and persistent proliferation of the MEE/MES	Xu et al., 2006
Tgfbr1 (Alk5) K14-Cre-mediated removal	Impaired palatal adhesion and fusion (partial) due to decreased MEE filopodia and to lack of apoptosis of the MES	Dudas et al., 2006
Tgfbr1 (Alk5) Wnt1-Cre-mediated removal	Increased apoptosis and cell proliferation in the palatal shelves. Anomalies in other skeletal craniofacial structures may also contribute to CP.	Dudas et al., 2006
Transcription factors		
Different compound mutants of *Alx4* and *Cart1*	Δ	Qu et al., 1999
Dlx1	Δ	Qiu et al., 1997
Dlx2	Δ	Qiu et al., 1997
Dlx5	Δ	Acampora et al., 1999; Depew et al., 1999
Foxc2 (previously *Mfh1*)	Δ(Craniofacial defects similar to those in *Gli2* mutants)	Lida et al., 1997
Foxe1(previously *Titf2*)	Palatal shelves elevate but fail to fuse with each other	De Felice et al., 1998
Foxf2	Δ?	Wang et al., 2003
Gli2	Δ	Mo et al., 1997
Gli3 xtJ	Δ	Mo et al., 1997
Hic1	Δ	Carter et al., 2000
Hoxa2	Δ	Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999
Myf5; MyoD	Primary palate and secondary palate do not fuse with each other	Rot-Nikcevic et al., 2005
Lhx8	Palatal shelves elevate but fail to make contact	Zhao et al., 1999
Msx1	Altered proliferation in palatal shelves	Satokata and Maas, 1994; Zhang et al., 2002
Osr2	Impaired proliferation and medio-lateral patterning in palatal shelves	Lan et al., 2004
p63	Altered epithelial–mesenchymal interactions. Palatal shelf epithelial differentiation defects?	Mills et al., 1999; Yang et al., 1999
Pax9	Δ	Peters et al., 1998
Pitx1	Δ	Lanctôt et al., 1999; Szeto et al., 1999

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Table 1 (*continued*)

Genetic loss-of-function	Causes of cleft palate	References
Transcription factors		
Pitx2	Palatal shelves elevate but are hypoplastic	Lu et al., 1999
Prx1 (previously *Mhox*)	Δ	Martin et al., 1995
Prx1; Prx2	Δ	ten Berge et al., 1998
Rae28	Δ	Takihara et al., 1997
Satb2	Patterning defects of the developing palate. Anomalies of other craniofacial structures may also contribute to the CP.	Dobreva et al., 2006
Sall3	Hypoplastic soft palate and epiglottis	Parrish et al., 2004
Shox2	Cleft of the anterior portion of the secondary palate due to abnormal proliferation and apoptosis.	Yu et al., 2005
Sim2	Palatal shelves are hypocellular and exhibit increased extracellular glycosaminoglycans	Shamblott et al., 2002
Sox9 haploinsufficiency	Δ	Bi et al., 2001
Tbx1	Δ	Jerome and Papaioannou, 2001
Cytoplasmic proteins		
Apaf1	Failure of fusion of palatal shelves owing to failure of apoptosis	Cecconi et al., 1998
Gad1	Delayed lifting of palatal shelves	Asada et al., 1997; Condie et al., 1997
3b-hydroxysterol-D7-reductase	Hypoplastic palatal shelves	Wassif et al., 2001
IKK1	Cleft palate	Li et al., 1999
p57kip2	Δ	Yan et al., 1997; Caspary et al., 1999
Viaat	Δ	Wojcik et al., 2006
Extracellular matrix components		
Col2a1	Δ	Pace et al., 1997
Perlecan	Δ	Arikawa-Hirasawa et al., 1999
Insertional mutations		
CASK (loss-of-function)	Δ	Wilson et al., 1993; Laverty and Wilson, 1998
Dlg (loss-of-function)	Δ	Caruana and Bernstein, 2001
Tbx10 (gain-of-function). *Dancer* mutation	Cleft lip and cleft palate due to ectopic expression of *Tbx10*	Bush et al., 2004
p23-Tbx10 transgenic mice	Cleft lip and cleft palate similar to that of *Dancer* mice	Bush et al., 2004

Δ Indicates cleft palate conditions that are or may be secondary to other craniofacial bone defects and/or hindrance by the tongue.

After vertical growth, the PS elevate into a horizontal position, and further extension allows contact between the opposing PS. Some genetic disruptions affect this second phase of PS growth. For instance, mice lacking *Tgfbr2* in the PS mesenchyme develop a CP due to reduced extension of the horizontal PS (Ito et al., 2003), and paracrine Tgfβ3 signaling in the PS mesenchyme seems to be required for this growth phase (Xu et al., 2006). Similarly, embryos lacking platelet-derived growth factor c (Pdgfc) activity show normal PS growth up to E13.5; however, after a delayed lifting, the hypoplastic PS are unable to abut (Ding et al., 2004). Loss of function of *single-minded2* (*Sim2*) in mice generates either a complete cleft of the secondary palate or a cleft of its posterior-most portion (Shamblott et al., 2002). The complete cleft seems to be caused by lack of outgrowth of the PS which are, however, able to elevate. The PS of $Sim2^{-/-}$ mice are hypocellular between E14.5 and E16.5, and histochemical staining suggested the presence of abnormally high amounts of hyaluronan (Shamblott et al., 2002). This aspect is interesting in light of the known role of hyaluronan (hyaluronic acid), a major component of the extracellular matrix, in regulating cell proliferation, differentiation and migration.

Several mutant mice display multiple craniofacial anomalies, where CP is one facet (Table 1). This constitutes a hurdle for the distinction between clefting due to endogenous anomalies within the palate and clefting secondary to malfunction and/or malformation of other structures. However, many of the targeted genes in those mouse models are expressed in the developing PS of wild-type embryos, implying intrinsic functions for these genes within the palate. Examples include *Pitx1* (Szeto et al., 1999), *Pitx2* (Lu et al., 1999), *Gli2* (Mo et al., 1997; Rice et al., 2006), *Ryk* (Halford et al., 2000), *Tbx1* (Jerome and Papaioannou, 2001; Zoupa et al., 2006), *Foxf2* (Wang et al., 2003), *Pdgf receptor a* (*Pdgfra*) (Soriano, 1997; Tallquist and Soriano, 2003). In the case of PdgfR-α, product of *Pdgfra*, there is recent evidence that the main action of this receptor in the PS mesenchyme is to mediate the paracrine function of the epithelially produced Pdgfc (Ding et al., 2004).

While the above mutations generate loss of gene function, the spontaneous mutation *Dancer* in mice, which generates CL/P, has been shown to cause ectopic expression of a variant *Tbx10* transcript in palate and lip primordia as well as in other structures. Furthermore, ectopic transgenic overexpression of *Tbx10* recapitulates the CL/P phenotype of *Dancer* mice (Bush et al., 2004). Thus, expression of *Tbx10* in forbidden territories is the cause of the clefting anomalies in *Dancer* embryos. How this gene affects palatogenesis remains to be elucidated.

Molecular control of palatal shelf elevation

Despite the availability of mutant mice with CP due to failure or delayed PS elevation, the exact mechanisms that bring the PS from a vertical to a horizontal position are still poorly defined. Various mechanisms have been postulated and remain basically unchanged since the subject was first reviewed by Lazzaro in 1940 and later by Ferguson in 1988. The general consensus is that PS elevation is a rapid movement, triggered by both intrinsic forces within the PS proper and by influences from other craniofacial and oral structures, including movement of the tongue, growth of the basicranium and mandible (Ferguson, 1988).

The role of steric hindrance by the tongue in preventing PS elevation and inducing CP is well illustrated in *Hoxa2*$^{-/-}$ mice. In these, an abnormal position of the tongue is caused by insertional defects of the hyoglossus muscle into the hyoid bone. The penetrance of CP was dramatically reduced when the tongue defect was rescued in compound mutants lacking both Hoxa1 and Hoxa2 function (Barrow and Capecchi, 1999).

The concept of rapid PS self-generating intrinsic "erectile" forces instigating their elevation was first suggested by Lazzaro (1940) based on observations of embryos with one PS horizontal and the other vertical. Lazzaro also suggested swelling of the PS due to increase in extracellular matrix as the causative factor. The role of the extracellular matrix in PS elevation has been supported and refined by further studies and is at present accepted as an important determinant of PS elevation. Those studies (reviewed in Ferguson, 1988) suggested that a progressive differential accumulation of glycosaminoglycans, primarily hyaluronan, in the PS plays a role in their elevation. Hyaluronan is a highly charged glycosaminoglycan that retains high amounts of water, thus forming hydrated gels leading to the expansion of the extracellular matrix. Other constituents of the PS such as collagen fibers, vascularization, the epithelial covering as well as polarized alignment of mesenchymal cells have also been suggested to contribute to the PS's intrinsic elevating force (Ferguson, 1988).

Early studies attributed a role to neurotransmitters in PS elevation (Ferguson, 1988). At present, it is widely accepted that the neurotransmitter γ-aminobutyric acid (GABA) regulates not only neuronal activities but also cell migration, survival, proliferation and differentiation in both neuronal and nonneuronal cells (Varju et al., 2001). Teratological studies in rodents showed that GABA or GABA agonists generate CP by inhibiting PS elevation, whereas GABA antagonists stimulate the process (Miller and Becker, 1975; Wee and Zimmerman, 1983). Presence of endogenous GABA or glutamic acid decarboxylase 67 (Gad 67 encoded by *Gad1*), one of GABA biosynthetic enzymes, has also been demonstrated in the PS (Wee et al., 1986; Asada et al., 1997; Hagiwara et al., 2003). The implication of GABA in palate development was further demonstrated by genetic studies in mice lacking the β3 subunit of $GABA_A$ receptor or lacking Gad67, which both develop a CP without other craniofacial malformations (Culiat et al., 1993; 1995; Asada et al., 1997; Condie et al., 1997; Homanics et al., 1997). The remarkable similarity in the CP phenotype between mutants lacking Gad67 and those deficient in $GABA_A\beta3$ indicates that GABA signaling through $GABA_A$ receptor is crucial for palatogenesis. In perinatal fetuses lacking Gad67 and $GABA_A\beta3$, the PS are elevated above the tongue. However, it is still not clear whether the CP was secondary to growth defects or to delayed elevation of the PS, as no survey of palate development at different stages was performed in those mutants. Since GABA is an important neurotransmitter in the brain, concerns were raised as to whether the CP in the above mutants was merely a secondary effect due to neuronal dysfunction. However, transgenic mice that had a normal neuronal $GABA_A\beta3$ but still lacked this receptor's function in the palate developed a CP (Hagiwara et al., 2003), implying a role for GABA signaling within the palate. Recently, inactivation of the murine neuronal vesicular inhibitory amino acid transporter (Viaat), which allows synaptic co-release of GABA and glycine, has been shown to generate a CP due to tongue immobility (Vojcik et al., 2006). This, however, does not exclude a function for GABA signaling within the palate proper. Both increased and decreased GABA signaling impinges upon palatogenesis. This indicates the requirement of a tight control of the amplitude of GABA signaling for an adequate development. Interestingly, significant associations between *GABRB3* (Scapoli et al., 2002) and *GAD1* (Kanno et al., 2004) and nonsyndromic CL/P have been recently reported in humans.

Delayed PS elevation occurs in *Osr2*$^{-/-}$ (Lan et al., 2004), *Pdgfc*$^{-/-}$ (Ding et al., 2004) and in *Dancer* (Bush et al., 2004) mutant mice. While altered mesenchymal proliferation patterns may underlie the delayed lifting of the shelves in those models, changes in extracellular turnover are also possible contributing factors that await further studies.

Molecular control of palatal shelf fusion

Fusion of the opposing PS is an important step during palatogenesis. This takes place by a sequence of events, including removal of the superficial flat periderm cells, contact and adhesion of the opposing MEE creating the MES, degeneration of the MES and, finally, mesenchymal confluence at the midline. Anteriorly, the PS fuse also with the nasal septum to form the nasopalatine junction and with the primary palate. Disappearance of the MES is necessary for a successful palatal fusion. Until recently (Vaziri Sani et al., 2005), the fate of the MES has been subject to considerable disagreements, and three mechanisms imparting the disappearance of the MES have been suggested: apoptosis, epithelial–mesenchymal transformation (EMT) and migration of MES cells towards the periphery of the midline.

Early and recent studies provided morphological and molecular evidence for the occurrence of apoptosis in the regressing MES (Glücksmann, 1951; Saunders, 1966; DeAngelis and Nalbandian, 1968; Smiley and Dixon, 1968; Shapiro and Sweney, 1969; Smiley and Koch, 1975; Mori et al., 1994; Tanigushi et al., 1995; Martínez-Álvarez et al., 2000a,b; Cuervo et al., 2002; Cuervo and Covarrubias, 2004; Vaziri Sani et al., 2005). However, others suggested that the cells of the MES as well as the cells of the epithelial seam along the nasopalatine

junction remain viable and undergo EMT, i.e., a transdifferentiation of MES cells into fibroblasts. These suggestions were based on morphological criteria and on cell tracking with lipophilic molecules (Fitchett and Hay, 1989; Shuler et al., 1991, 1992; Griffith and Hay, 1992; Nawshad and Hay, 2003; Nawshad et al., 2004; Hay, 2005). EMT of the MES has been proposed as the major mechanism underlying the disappearance of the MES to generate mesenchyme continuity, thus preventing palatal clefting (Nawshad and Hay, 2003; Nawshad et al., 2004; Hay, 2005). The establishment of the concept of EMT as the prevailing mechanism of MES disappearance led to several studies attributing roles to different molecules in mediating EMT, including Tgfβ3, Lef1, Smads, RhoA, phosphatidylinositol 3-kinase, matrix metalloproteinases (MMPs), and Snail (Kaartinen et al., 1997, 2002; Sun et al., 1998; Blavier et al., 2001; Kang and Svoboda, 2002, 2005; Nawshad and Hay, 2003; Dudas et al., 2004a; Martínez-Álvarez et al., 2004; Nawshad et al., 2004; Hay, 2005).

However, a recent study (Vaziri Sani et al., 2005) using genetic marking of *Shh*- and *keratin-14*-expressing palatal epithelial cells and their progeny ruled out the occurrence of EMT during PS fusion with each other and with the nasal septum (see also Fig. 2D). In addition to the reliability provided by the use of both the K14-Cre- and the Shhgfp-Cre-mediated genetic marking in the fate-mapping of the MES, special care was taken to preserve the morphological integrity of PS mesenchyme and to avoid the use of thick, overstained sections which may lead to masking of any lacZ-negative epithelial cells (Vaziri Sani et al., 2005). These criteria were not met in a recent study (Xu et al., 2006). The findings ruling out the occurrence of EMT during palatal fusion thus imply that while the abovementioned factors may have a role in palate fusion, as they are expressed and activated during this developmental stage, it is not to regulate EMT. Some proponents of the EMT concept reject the involvement of apoptosis in the regressing MES based on the fact that in studies marking apoptotic cells, the majority of the MES displays a healthy look with only a few cells showing apoptotic features (Kang and Svoboda, 2005). However, it should be kept in mind that regression of the MES is a progressive, yet rapid event, and a synchronized massive cell death along the MES would be detrimental to palatal fusion. The progressive nature of MES regression is well portrayed by the presence of epithelial remnants of the MES that are located at different dorso-ventral and anterior–posterior levels at the midline as well as along the nasopalatine junction (Vaziri Sani et al., 2005; Figs. 2D, J). The crucial role for apoptosis during palatal fusion is demonstrated in mice lacking the function of the apoptotic protease activating factor 1 (Apaf1), which display a CP due to persistence of the MES (Cecconi et al., 1998). More recent experimental studies in vitro point to the requirement of apoptosis for palatal fusion, a process that is likely regulated by retinoids (Cuervo et al., 2002).

Migration of cells of the MES along the midline towards the oral and nasal epithelia has also been suggested as an alternative mechanism underlying MES regression (Carette and Ferguson, 1992). However, a recent study (Cuervo and Covarrubias, 2004) showed that the cells which migrate upon PS contact to form epithelial triangles along the midline within the oral and nasal epithelia are those of the periderm that cover the MEE. Those findings are also contrasting with previous studies suggesting shedding of periderm cells before PS contact (Fitchett and Hay, 1989). It seems also that peridermal cells are necessary for establishing the first contact between the opposing PS, and that their migration away from the midline is necessary for triggering apoptosis in both the MES and periderm cells (Cuervo and Covarrubias, 2004). These aspects need to be further studied with specific markers of the periderm.

Targeted gene ablation in mice identified several factors playing a determinant role in palate fusion. These include Tgfβ3 (Kaartinen et al., 1995; Proetzel et al., 1995), the forkhead domain-containing transcription factor Foxe1 (previously TTF-2; De Felice et al., 1998), epidermal growth factor receptor (EgfR; Miettinen et al., 1999) and Pdgfc (Ding et al., 2004). Loss of function of these factors generates CP with no or minor other craniofacial anomalies. In vitro explant cultures showed that PS from *Tgfβ3*, *Egfr* and *Pdgfc* mutants fail to fuse owing to failure of the MES to degenerate (Kaartinen et al., 1995; Miettinen et al., 1999; Ding et al., 2004). Importantly, studies in humans identified a mutation within the forkhead domain of *FOXE1* in siblings with thyroid agenesis, CP and choanal atresia (Clifton-Bligh et al., 1998) and associated *TGFB3* with nonsyndromic CP (Lidral et al., 1998).

Cell–cell junctional complexes are essential for cell survival, morphogenesis, proliferation and differentiation. Adherens junctions (AJs) are key structures for cell–cell adhesion. They contain at least two types of cell adhesion molecules (CAMs), cadherins and nectins (Tachibana et al., 2000). In epithelial cells, α-catenin functions as a molecular switch that regulates actin filament assembly at sites of E-cadherin-mediated cell–cell adhesion (Gates and Peifer, 2005). Nectins are immunoglobulin-like CAMs belonging to a family of four members and are linked to the actin cytoskeleton through afadin. Accumulating evidence indicates that nectins first bring about cell–cell adhesion and thereafter recruit cadherins to the nectin-based adhesion sites through afadin and catenins (Irie et al., 2004). Adhesion of the opposing MEE is an important step of palatogenesis. In both human and mouse embryos, *E-cadherin* is expressed in epithelia covering the frontonasal and medial nasal processes as well as during the different stages of palate development, including in the epithelial islands, remnants of the MES (Montenegro et al., 2000; Tudela et al., 2002; Vaziri Sani et al., 2005; Frebourg et al., 2006). Targeted mutation of *E-cadherin* in mice is incompatible with development beyond the morula stage and morula cells dissociate shortly after compaction (Riethmacher et al., 1995). However, mutations of *CDH1/E-cadherin* which delete the extracellular cadherin repeat domains required for cell–cell adhesion have been recently associated with CL/P in families with hereditary diffuse gastric cancer (Frebourg et al., 2006). E-cadherins are known to form dimers, indicating that the mutant proteins might have transdominant negative effects over the wild-type proteins (Frebourg et al., 2006).

Mutations of the poliovirus receptor related-1 (*PVRL1*) gene encoding nectin1 cause the autosomal recessive syndrome CL/

P-ectodermal dysplasia1 (CLPED1) which includes Zlotogora-Ogür syndrome and Margarita Island ectodermal dysplasia (Suzuki et al., 2000) and seem to constitute a genetic risk factor for nonsyndromic CL/P (Suzuki et al., 2000; Scapoli et al., 2006). The clinical features of CLPED1 include CL/P, tooth and hair anomalies, mid-facial hypoplasia, limb anomalies and sometimes mental retardation (Rice, 2005). As expected, *nectin1* is co-expressed with E-cadherin in epithelia, including the palatal MEE in both human and mouse embryos (Suzuki et al., 2000; Ding et al., 2004). However, mice deficient in either *nectin1*, *nectin2 or nectin3* do not display defects in AJs and tight junctions in most tissues where nectins are expressed, and *nectin1*-deficient mice do not develop CP. This might be a consequence of functional redundancy of each nectin in these tissues (Irie et al., 2004 and references therein).

The exact cellular alterations leading to CL/P following mutations of *CDH1/E-cadherin* and *PVRL1* are still not well defined. The cleftings might be caused by impaired epithelial differentiation and integrity and/or loss of the adhesive functions of the lip and palatal epithelia. Although altered lip and palatal primordia fusion is likely the cause of clefting in the absence of nectin1 and E-cadherin, there is a need for animal models and further studies to elucidate the role of these molecules in lip and palate development.

During the last few years, extensive efforts have been made to shed light upon the role of Tgfβ3 during palatal fusion. Adhesion of the MEE upon PS contact is a necessary step for fusion. Tgfβ3, which is expressed in the MEE before and during PS fusion, has been shown to mediate MEE adhesion of the opposing PS through filopodia (Taya et al., 1999, Martínez-Álvarez et al., 2000a) and chondroitin sulfate proteoglycans (Gato et al., 2002) at the apical surface of MEE cells and to be necessary for apoptosis of the regressing MES (Martínez-Álvarez et al., 2000b, 2004). Importantly, in the absence of Tgfβ3, MEE cells display altered distribution of E-cadherin, α- and β-catenins and impaired cell–cell adhesion (Tudela et al., 2002). Early studies on fusion processes in different systems consistently show the presence of filopodia at the tip of fusing epithelial sheets (Fristrom, 1988). More recent studies indicate that E-cadherin is required for fusion, whereas filopodia seem to be crucial for proper alignment and guidance of cell sheets that are fated to fuse, but not for the fusion process itself (Schöck and Perrimon, 2002). Thus, Tgfβ3 plays a crucial role during the different steps of MEE adhesion and fusion.

Other studies implicated Tgfβ3 in controlling the remodeling of the extracellular matrix through regulation of the expression of *Mmp13*, *Mmp2* and *Tissue inhibitor of metalloproteinase-2* (Blavier et al., 2001). These studies indicate that Tgfβ3 signaling operates not only in the MEE, but is also involved in mediating epithelial–mesenchymal interactions leading to tissue changes that regulate palatal fusion. The effects of Tgfβ3 on MES regression seem to be mediated by the Tgfβ type II and the Tgfβ type I receptor (Alk5)/Smad pathway as shown by loss and gain of function studies in vitro and in vivo (Dudas et al., 2004a, 2006; Cui et al., 2005; Xu et al., 2006). However, *Alk5* is not expressed in the MEE of the posterior palate just before and after fusion (Dudas et al., 2004a). As a consequence, posterior palate fusion of palatal explants was not inhibited following inactivation of Alk5 in vitro, whereas fusion was inhibited in the anterior palate (Dudas et al., 2004a). These findings are at odds with a recent study showing cleft of the posterior palate and superficial adherence of the middle and anterior palate following genetic ablation of *Alk5* in the palatal epithelium (Dudas et al., 2006). It is possible, however, that *Alk5* is expressed at low levels in the posterior palate, and that adenovirus-mediated expression of a dominant-negative *Alk5* in vitro is not sufficient to abrogate its activity as in the genetic in vivo system. While ablation of *Tgfbr2* in the PS mesenchyme generates CP secondary to abnormal mesenchymal proliferation (Ito et al., 2003), K14-Cre-mediated removal of this receptor activity in the palatal epithelium generates cleft of the soft palate and submucous cleft palate (Xu et al., 2006). The CP in mice lacking epithelial *Alk5* and *Tgfbr2* is secondary to persistence of the MES owing to failure of cells to undergo apoptosis (Dudas et al., 2006; Xu et al., 2006). In addition, continued abnormal proliferation of the MEE in the *Tgfbr2* deficient palatal epithelium generates epithelial overgrowth that hinders palatal fusion (Xu et al., 2006).

While inactivation of epithelial *Alk5* or *Tgfbr2* generates partial CP (Dudas et al., 2004a, 2006; Xu et al., 2006), *Tgfβ3* mutants display either a complete or partial secondary CP (Kaartinen et al., 1995; Proetzel et al., 1995). Furthermore, MEE-driven transgenic expression of Smad2 in a *Tgfβ3* null background has been shown to rescue the CP only partially, with the anterior-most and posterior-most segments remaining cleft (Cui et al., 2005). These differences may be attributed to deficient Tgfβ3 paracrine signaling that is required for mesenchymal proliferation (Xu et al., 2006) and/or for the induction of other mesenchymal factors necessary for epithelial remodeling in the *Tgfβ3* mutant model and which are maintained in mutants lacking epithelial *Alk5* and *Tgfbr2*. Another parsimonious explanation is that some epithelial cells escape K14-Cre-mediated ablation of *Alk5* and *Tgfbr2*. Interestingly, loss of epithelial TgfβRII activity is followed by reduction of the expression of *Irf6* and *Mmp13* in the MEE (Xu et al., 2006), in agreement with previous studies showing a key role for Tgfβ3 in the induction of these factors in the MEE (Blavier et al., 2001; Knight et al., 2006). In humans, *IRF6* mutations cause CL/P or CPO in Van der Woude syndrome and are also found in isolated CL/P and CPO (Kondo et al., 2002; Rice, 2005). Interestingly, IRFs and Smads have been proposed to share a conserved transactivating domain (Eroshkin and Mushegian, 1999), and interferon-γ has been shown to inhibit Tgfβ/Smad signaling (Ulloa et al., 1999), suggesting interactions of the two pathways.

Under normal conditions, PS epithelia do not fuse with other oral structures. However, in the absence of Fgf10 PS epithelia fuse with the tongue and mandible (Rice et al., 2004; Alappat et al., 2005) at sites of increased apoptosis (Alappat et al., 2005). These anomalies have been suggested to be caused by a severe reduction of the expression of *Jagged2* (*Jag2*), encoding a ligand for the Notch family receptors and to ectopic *Tgfβ3* production in the oral and nasal epithelia (Alappat et al., 2005). These assumptions are reasonable, given the well established

role for Tgfβ3 in palatal fusion and the phenotype of embryos lacking *Jag2*, which display a CP associated with aberrant fusion of the PS with the tongue and mandible (Jiang et al., 1998). More recent evidence from analyses of *Jag2* mutant embryos indicates that the Jag2-Notch signaling acts primarily by preventing inappropriate PS adhesion to other oral epithelia through control of oral epithelial differentiation (Casey et al., 2006). In contrast to *Fgf10* mutants, no ectopic expression of *Tgfβ3* or its target, *Mmp13*, was documented in the *Jag2* mutant oral structures (Alappat et al., 2005; Casey et al., 2006). These data indicate that the ectopic function of Tgfβ3 alone cannot account for the aberrant epithelial adhesions between the PS and other oral structures. Importantly, the Jag2-Notch signaling was found to be attenuated in the PS epithelia of wild-type embryos as compared to the rest of the oral epithelium, thus explaining the normal differentiation of PS epithelia in the *Jag2* mutants (Casey et al., 2006). These results raise the question of whether abnormal activation of the Jag2-Notch signaling in the MEE would prevent PS adhesion. Interestingly, aberrant fusions between the PS and tongue or PS and mandible have been described in human embryos and in teratological studies in the rat (Casey et al., 2006 and references therein). The Fgf and Jag2/Notch pathways might thus be implicated in those anomalies.

While both Pdgfa and Pdgfc are expressed in the PS epithelium, they act on the PS mesenchyme via their PdgfR-α. However, Pdgfc function within the palate seems to be nonredundant, as Pdgfa in the MEE was unable to rescue the clefting in *Pdgfc*$^{-/-}$ mice, and loss of Pdgfa alone does not generate CP (Ding et al., 2004 and references therein). Despite a failed fusion, the PS of *Pdgfc* mutant mice displayed normal expression patterns of *Tgfβ3*, *Irf6* and *Pvrl1*. Conversely, *Tgfβ3*$^{-/-}$ palates exhibited normal Pdgfc expression (Ding et al., 2004). These important findings unveil Pdgfc signaling as a new and independent pathway mediating epithelial–mesenchymal interactions during palatal fusion. Which signals trigger expression of *Pdgfc* in the epithelium and *Pdgfra* in the mesenchyme, which factors regulate the processing enzyme that activates the latent form of Pdgfc, and what are the targets of this signaling pathway? Future studies with this new model will certainly identify new players in palatogenesis.

Mutants lacking *Lhx8* develop CP without other craniofacial anomalies (Zhao et al., 1999). In these, the PS show normal proliferation and elevate on time but do not make contact and fuse. It has been suggested that mesenchymal Lhx8 activity may mediate epithelial–mesenchymal interactions that are crucial for PS fusion (Zhao et al., 1999). A first step towards addressing this issue requires *Lhx8*$^{-/-}$ PS explant cultures in vitro to determine whether they fuse or not.

Regionalization of the developing palate along the medio-lateral and anterior–posterior axes

The PS display a medio-lateral (ML) regional specification (Fig. 1F) which is translated morphologically into regional differentiation of the epithelium. The PS epithelia thus differentiate into a pseudostratified columnar ciliated epithelium on the nasal/medial side, a stratified squamous epithelium with the formation of rugae palatinae on the oral/lateral side and, finally, into a MEE at the tip of the PS which is fated to disappear upon PS fusion (Figs. 2I, J). The developing palate also displays regional differences along the anterior–posterior (AP) axis. Early studies documented the existence of higher amounts of hyaluronan in the anterior palate and in the lateral half of the PS than in the posterior palate and medial aspect of the PS, respectively (Knudsen et al., 1985; Brinkley and Morris-Wiman, 1987; Ferguson, 1988).

Interestingly, these palatal regional differences are back into focus as a number of genes have been found to be expressed differentially along the ML and AP axes (Hilliard et al., 2005). While ML differential gene expression patterns could be taken as an indication of early events regulating the fate of the PS epithelia, they might also underlie morphogenetic events necessary for palatal shelf growth and/or elevation. In this respect, a recent study (Lan et al., 2004) provided evidence for the role of Osr1 and Osr2 in controlling the ML differential proliferation of PS mesenchymal cells, necessary for PS growth and elevation to occur on schedule. First, *Osr2* transcripts were found throughout the palatal mesenchyme but showed a preferential accumulation in the lateral half of the PS. In contrast, starting at E13.5, *Osr1* was expressed virtually only in the proximo-lateral regions of the PS. These expression patterns underlie the unexpected preferential reduction of cell proliferation in the medial half of the PS of *Osr2* null mice (Lan et al., 2004). The PS of wild-type embryos display differential growth rates medio-laterally, being faster in the medial than in the lateral halves (Lan et al., 2004). Interestingly, *Pax9* which encodes a member of the paired class of transcription factors necessary for palate development (Peters et al., 1998), was found to display Osr2-dependent dynamic expression patterns in the PS mesenchyme (Lan et al., 2004). Thus, it seems that ML dynamic molecular changes occur at the time preceding palatal shelf elevation, and that *Osr* genes play a crucial role in these patterning events.

Elevation of the PS occurs in an AP sequence, and the horizontal PS approximate and fuse with each other first at the level of the second rugae, thereafter fusion proceeds anteriorly and posteriorly (Ferguson, 1988). However, this sequence of palatal closure does not imply that fusion of the soft palate is dependent on that of the hard palate. In fact, a rare condition in which the soft palate is intact whereas the hard palate is cleft has been reported in both humans and animals (Schüpbach, 1983; Yu et al., 2005), implying that closure events anteriorly and posteriorly are not dependent on one another. Interestingly, *Shox2/SHOX2* was expressed solely in the presumptive hard palate in both human and mouse embryos, which would be consistent with the palatal phenotype of *Shox2*$^{-/-}$ mice (see above). Recombination studies showed that the restricted expression of *Shox2* anteriorly depends on signals produced by the epithelium of the anterior palate (Yu et al., 2005).

Other factors involved in palatogenesis, including *Bmp2*, *Bmp4*, *Msx1* and *Fgfr2b* have been found to exhibit differential expression patterns along the AP axis of the developing palate. In addition, explant experiments have shown that the anterior and posterior palatal mesenchymes show different molecular

and cellular responses to growth factors (Hilliard et al., 2005). In the developing mouse palate, expression of *Tbx22* has been shown to be restricted posteriorly, in a region encompassing both the soft palate and the posterior-most part of the hard palate (Hilliard et al., 2005; AGL unpublished). This AP pattern, which is not related to the specification of the hard and soft

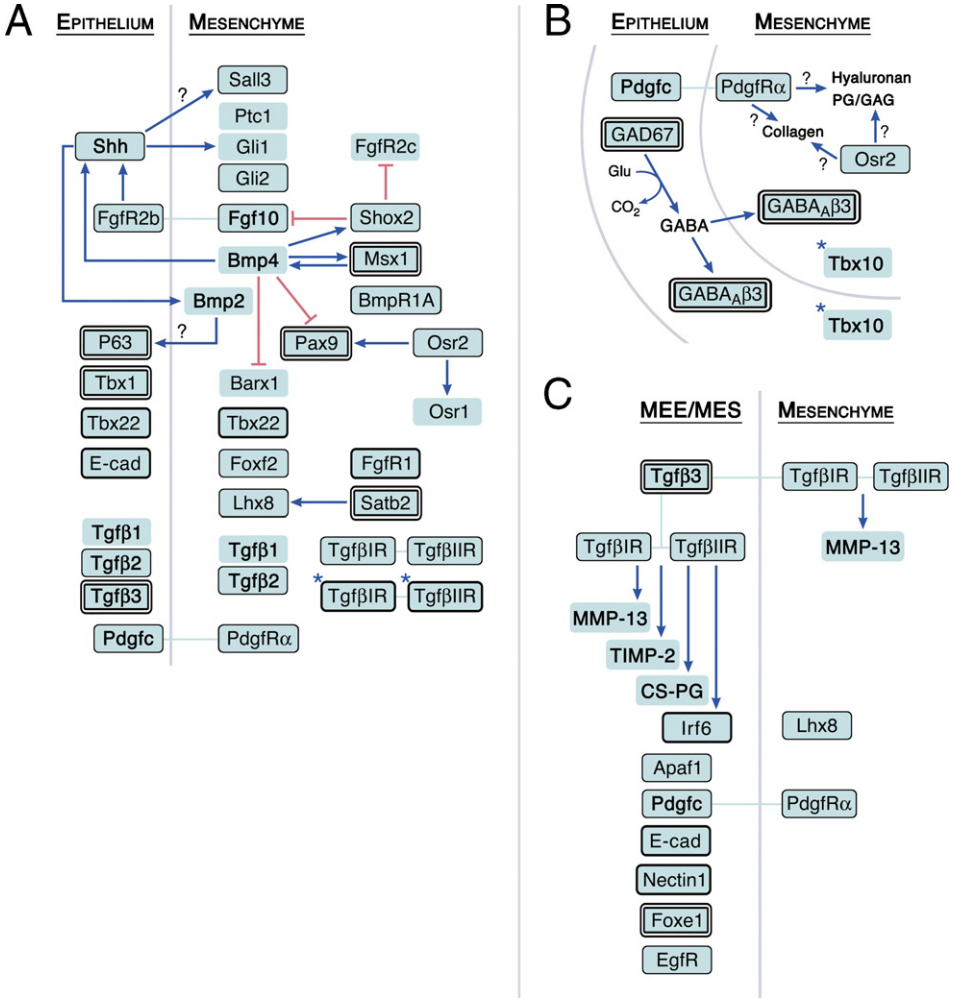

Fig. 4. Molecular signalings mediating epithelial–mesenchymal interactions during palatal shelf (PS) growth (A), elevation (B) and adhesion/fusion (C). Mutations of genes encoding most of the factors shown induce cleft palate in mice (thin frame) or are implicated in palatal clefting in humans (thick frame). Those implicated in palatal clefting in both mice and humans are indicated by a double frame. Secreted proteins (bold letters) operate within their site of production and/or cross the basement membrane separating the epithelium (E) and mesenchyme (M) and act in the adjacent tissue layer by binding to their receptors. Stimulatory and inhibitory activities are indicated by blue arrows and red bars, respectively. Binding of signaling molecules to their receptors is represented by thin lines. Shh may regulate expression of *Sall3* in the palatal M. Fgf10 from the M is necessary for induction of *Shh* expression in the E. In turn, Shh stimulates a mitogenic response in both the E and M and prevents apoptosis in the E (not represented). Bmp4 functions in an autoregulatory loop with Msx1 in the M and is necessary for epithelial *Shh* expression. Shh-induced Bmp2 stimulates mesenchymal cell proliferation. Bmp4 activity modulates the expression of *Barx1* and *Pax9* and is necessary for maintaining the expression of *Shox2*. Shox2 activity prevents expression of *Fgf10* and *FgfR2c* at ectopic sites. Satb2 activity regulates *Lhx8* expression. p63 is necessary for epithelial integrity that is crucial for adequate E–M interactions and its expression may be regulated by Bmps. Tgfβs emanating from both the E and M signal through TgfβIIR and TgfβIR heterotetramers to elicit mesenchymal cell proliferation. The asterisks indicate that in humans, mutations of genes encoding TGFβRI and TGFβRII leading to abnormally increased Tgfβ signaling are associated with CP. The schematic in panel A does not take into account the regionalized expression patterns of some factors along the medio-lateral and anterior–posterior axes of the growing PS. Several factors have been suggested to be involved in palatal shelf elevation (B). Hyaluronan forms gels after binding water and elicits tissue expansion of the PS. Hyaluronan also regulates cell proliferation and migration. Proteoglycan–glycosaminoglycans (PG–GAG) seem to be crucial for proliferation and polarized alignment of PS mesenchymal cells. Collagen fibers may also control cell alignment. γ-Aminobutyric acid (GABA), synthesized from glutamic acid (Glu) by glutamic acid decarboxylase 67 (GAD67) binds to $GABA_A$ receptor β3 subunit ($GABA_Aβ3$). GABA signaling may elicit a range of biological activities, including cell proliferation and migration necessary for PS lifting. Pdgfc and Osr2 may be involved in regulating extracellular matrix composition. Ectopic activation of Tbx10 (asterisk) seems to delay PS elevation in mice. Adherence and fusion of the opposing PS are also under a tight molecular control (C). Tgfβ3 produced by the MEE induces the accumulation of chondroitin sulfate proteoglycans (CS-PG) at the apical surface of MEE cells and is necessary for the expression of genes encoding Irf6, MMP-13 and TIMP-2. Tgfβ3 also induces apoptosis in the MES (not represented). Apaf1 is a key regulator of apoptosis in the MES. Pdgfc produced by the MEE signals through PdgfRα in the M. In turn, unknown Pdgfc-dependent factors in the M signal back to the MEE/MES to induce its degeneration. E-cadherin (E-cad) and nectin1 are crucial for cell–cell adhesion and epithelial integrity and may be necessary for initial adhesion of the opposing MEE. Lhx8 may be required for palatal fusion. The regulation and downstream targets of most of the factors involved in palate development are however yet to be determined. Fgf, Pdgfc, Bmp and Tgfβ3 signaling involves ligand binding to their cognate receptors which consist of homodimers, heterodimers or heterotetramers. These aspects have not been depicted in panels A, B or C for the sake of simplicity.

palates, instead reflect molecular heterogeneities which may have other functional implications in palatal growth.

The above observations point to the existence of a molecular regionalization within the developing palate. This implies that mesenchymal and epithelial cells within the PS have different histories and thus may respond differently to identical inputs. Keeping these aspects in mind, information gathered from scrutiny of gene expression patterns along the AP and ML axes of key players in palate development will certainly help us understanding the molecular and cellular interactions that take place during the different stages of palate development.

Concluding remarks

Needless to say, there is a constellation of molecules that are dispatched and engaged in complex interactions to drive palate development in a concerted mode of action (Fig. 4). Some of the factors act as repressors, others as activators, whereas some display dual effects depending on the temporal and spatial context. Some genes are 'guilty by association' as mutations of upstream regulators lead to their misexpression. Information from mouse models point to the importance of well balanced, spatiotemporally controlled molecular activities, as both deficiencies and overactivations orthotopically or ectopically impede development. These mouse models are precious as they offer us the opportunity to continuously probe for new factors implicated in normal and abnormal palate development. There is no doubt that major advances have been achieved and contributed to a better understanding of the molecular and cellular mechanisms that regulate palatogenesis and cause CP. However, we still know relatively little about the regulation and targets of many molecules identified as playing pivotal roles during palatogenesis (Fig. 4). Also knowing target genes of key regulators of palate development ensures the identification of novel risk factors for CP. Careful in vitro functional studies provide a wealth of information. In this respect, many factors shown in early meticulous in vitro studies (Ferguson, 1988), before the 'molecular era', to be crucial for palatogenesis turned out to be involved in palate clefting in both human and mice. Several different pathways elicit the same cellular responses. However, many signaling factors such as secreted proteins seem to be functionally obligatory and nonredundant, since removal of a single, specific factor may lead to dramatic defects. It is still unclear how and at which level pathways regulating the same or antagonistic cellular responses intersect, and addressing these issues constitutes a challenge for future studies. The interactions between environmental factors and genes in the ethiopathogenesis of CP are another important facet that requires further efforts. Fortunately, amazing advances have been and continue to be achieved in different fields providing state of the art research tools that are being used to unveil more secrets of palate development and clefting.

Acknowledgments

I am grateful to Dr. Anders Linde for helping with the figures. A.G.L. is supported by the Swedish Research Council-Medicine (grants 2789 and 15181), the Thuréus, the Åke Wiberg and the W and M Lundgren Foundations, as well as by the Department of Odontology at Sahlgrenska Academy.

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